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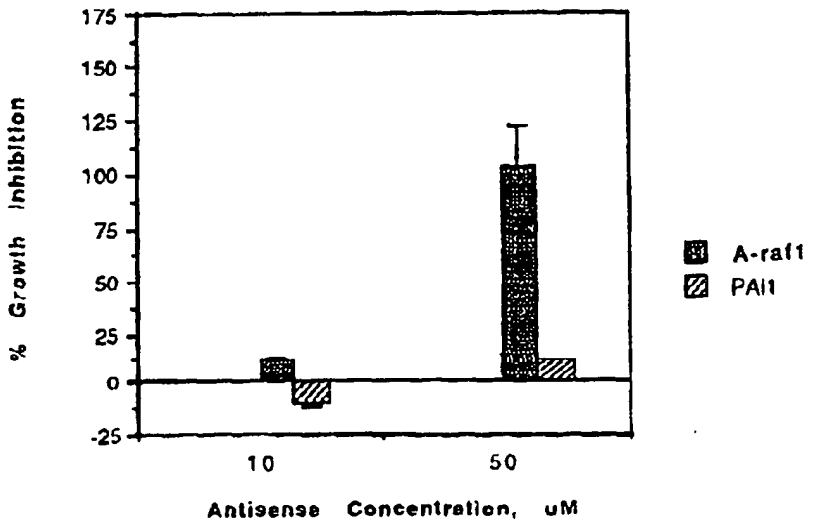

(57) Abstract

The present invention is directed to a polynucleotide, of about 10 to about 50 nucleic acid bases in length, which hybridizes to the gene encoding *raf*. The present invention is also directed to a pharmaceutical composition comprising the above polynucleotide dissolved or dispersed in a physiologically tolerable diluent. The present invention is further directed to a process for inhibiting vascular smooth muscle cell proliferation, a process for treating vascular smooth muscle cell proliferation, and process for treating a disease state involving smooth muscle cell proliferation. These processes comprise contacting the vascular smooth muscle cells or administering to a host mammal in need of such treatment, an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for *raf*.

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ANTISENSE MOLECULES DIRECTED AGAINST GENES OF THE RAF ONCOGENE FAMILY

DESCRIPTION

Cross Reference to Related Application

This application is a continuation-in-part of U.S. Application Serial No. 07/999,710, filed December 31, 1992.

TECHNICAL FIELD

The present invention relates to growth-factor related polynucleotides and their use in inhibiting the proliferation of smooth muscle cells, and more specifically to antisense molecules corresponding in sequence to portions of the gene for the oncogene raf, including A-raf-1 B-raf-1 and C-raf-1, and their use in inhibiting the proliferation of smooth muscle cells.

BACKGROUND OF THE INVENTION

Antisense polynucleotides contain artificial sequences of nucleotide bases complementary to messenger RNA (mRNA or message) or the sense strand of double stranded DNA. Admixture of sense and antisense oligo- or polynucleotides under appropriate conditions leads to binding of the two molecules, or hybridization.

When these polynucleotides bind to (hybridize with) mRNA, inhibition of translation occurs. When these polynucleotides bind to double stranded DNA, inhibition of transcription occurs. The resulting inhibition of translation and/or transcription leads to an inhibition of the synthesis of the encoded protein such as the proteins of the tissues, and more importantly here, various cellular growth factors, growth factor receptors, and oncogenes (many of which act as growth factors, receptors or mediators of signal transduction).

There are several members of the raf oncogene family, including the genes for A-raf-1, B-raf-1 and C-raf-1. The products of the raf gene family, including A-raf-1 and C-raf-1 are key molecules in growth factor signal transduction (J. Biol. Chem. 268: 7338-7345 (1993)). Excessive growth factor signalling is a common phenomenon in cardiovascular disease. Growth factors bind their cognate receptors and thereby stimulate receptor tyrosine kinase activity. Down-stream signals impinge on raf kinases or serine/threonine specific protein kinases. These in turn activate additional kinases that eventually stimulate transcription factor phosphorylation and are thus the final step in mitogenic signalling.

Activated smooth muscle cells (SMC) elaborate growth factors such as platelet derived growth factor (PDGF), basic and acidic fibroblast growth factor, interleukins and transforming growth factor β. Likewise, the SMC increase the production of PDGF receptor, FGF receptor, and epidermal growth factor receptor.

Activation of SMC, leading to the proliferation of those cells, occurs in response to a number of stimuli, including surgical procedures such as coronary angioplasty. The proliferation of SMC results in such disease states as atherosclerosis and restenosis.

An in vitro assay system has been developed to study smooth muscle cell proliferation. This assay system is considered to be a useful model for SMC proliferation in vivo. Gordon et al. have shown that SMC proliferation results from aortic and carotid balloon catheter injury, and is a result of atherosclerosis, providing a positive correlation

between SMC proliferation and stenosis. Gordon et al., Proc. Natl. Acad. Sci. USA 87:4600-4604 (1990).

Speir et al. have studied the inhibition of SMC proliferation in vitro by using an antisense polynucleotide to proliferating cell nuclear antigen (PCNA). However, these workers could not inhibit proliferation below 50 %, and the inhibition required high levels of the 18-mer antisense oligonucleotide used in those studies.

This invention demonstrates the biological action of antisense polynucleotides directed against genes of the raf oncogene family, including A-raf-1, B-raf-1 and C-raf-1 as useful for anti-proliferative activity against smooth muscle cell proliferation. This invention is applicable to a number of disease states in which the proliferation of smooth muscle cells is involved, including, but not limited to, vascular stenosis, post-angioplasty restenosis (including coronary, carotid and peripheral stenosis), other non-angioplasty reopening procedures such as atherectomy and laser procedures, atherosclerosis, atrial-venous shunt failure, cardiac hypertrophy, vascular surgery, and organ transplant.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to a polynucleotide of about 10 to about 50, preferably about 15 to about 25, and more preferably about 20, nucleic acid bases in length, which polynucleotide hybridizes to the gene encoding raf, including A-raf-1, B-raf-1 and C-raf-1. A preferred polynucleotides is an antisense molecule having the sequence shown in SEQ ID NO:1.

GCCCCGTGGTGGCTCCAT (SEQ ID NO:1)

Further preferred polynucleotides include

ACCGCTCAGCGCCGCCAT (SEQ ID NO:2),

TCCCTGTATGTGCTCCAT (SEQ ID NO:3).
AGGGGGGCCCCGTGGTGGCTCCAT (SEQ ID NO:4),
GGGGCCCCGTGGTGGCTCCATGGA (SEQ ID NO:5),
AGGGGGGCCCCGTGGTGGCTC (SEQ ID NO:6),
GGGGCCCCGTGGTGGCTCCAG (SEQ ID NO:7),
GCCCCGTGGTGGCTCCATGGA (SEQ ID NO:8),
GCCCCGTGGTGGCTCCATGGAGCC (SEQ ID NO:9),
GCACATGGGGACCCCCTC (SEQ ID NO:10),
GCCCTCACACCACTGGGT (SEQ ID NO:11),
GCCCTCGCACCACTGGGT (SEQ ID NO:12),
CCGGCGCCGGCCTCGGGC (SEQ ID NO:13), and
GCATTCTGCCCCCAAGGA (SEQ ID NO:14).

In a preferred embodiment, the bases of the polynucleotide molecule are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease and/or endonuclease enzymes. Preferred psuedophosphate bonds are phosphorothioate bonds.

The present invention is further directed to a polynucleotide of about 10 to about 50, preferably about 20 to about 40, and more preferably about 20, nucleic acid bases, which polynucleotide hybridizes to the about 5 to about 25, preferably about 10 to about 20, and more preferably about 10, nucleic acid bases flanking the start codon of the mRNA for raf, including A-raf-1, B-raf-1 and C-raf-1.

A preferred such polynucleotide is an antisense molecule having the sequence shown in SEQ ID NOs 1 through 9 above.

The present invention is also directed to a pharmaceutical composition comprising a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf, dissolved or dispersed in a physiologically tolerable diluent.

The present invention is further directed to a process for inhibiting vascular smooth muscle cell proliferation that comprises contacting vascular smooth muscle cells whose growth is to be inhibited in an aqueous medium suitable for growth of those cells with an inhibition-effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf and maintaining said contact in said aqueous medium under biological culture conditions for a time period sufficient for the growth of the contacted cells to be inhibited.

The present invention is still further directed to a process for treating vascular smooth muscle cell proliferation that comprises administering to a host mammal in need of such treatment an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

The present invention is yet further directed to a process for treating a disease state involving the proliferation of vascular smooth muscle cells that comprises administering to a host mammal in need of such treatment an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 shows the percentage of growth inhibition of smooth muscle cells upon the addition of either 10 μM or 50 μM of antisense polynucleotides directed against the gene for human A-raf-1 and the gene for plasminogen activator inhibitor 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a polynucleotide of about 10 to about 50 nucleic acid bases in length, which polynucleotide hybridizes to the gene for raf.

The gene for raf can be derived from any mammal, including mouse and humans. Preferably, the gene for raf is that of human raf.

The polynucleotide may preferably be from about 15 to about 25 nucleic acid bases in length, and more preferably about 20 nucleic acid bases in length.

The present invention is further directed to a polynucleotide of about 10 to about 50, preferably about 20 to about 40, and more preferably about 20, nucleic acid bases, which polynucleotide hybridizes to the about 5 to about 25, preferably about 10 to about 20, and more preferably about 10, nucleic acid bases flanking the start codon of the mRNA for raf.

It is to be understood that the present invention contemplates a polynucleotide that hybridizes to any part of the gene for any member of the raf oncogene family that is capable of inhibiting the proliferation of smooth muscle cells.

As used herein, "polynucleotide" refers to a covalently linked sequence of nucleotides in which the 3′ position of the pentose of one nucleotide is joined by a phosphodiester group to the 5′ position of the pentose of the next nucleotide. The nucleotides may be composed of deoxyribonucleotides or ribonucleotides.

Polynucleotide hybridization of greater than about 90 percent homology (identity), and more preferably about 99 percent homology, is contemplated in the present invention.

A. The Polynucleotides

A preferred polynucleotide is an antisense molecule having the sequence shown in SEQ ID NO:1, directed against position +1 to +18 relative to the start codon for the gene for human A-raf-1.

GCCCCGTGGTGGCTCCAT (SEQ ID NO:1)

Further preferred polynucleotides include

ACCGCTCAGCGCCGCCAT (SEQ ID NO:2), directed against positions +1 to +18 relative to the start codon for the gene for human B-raf-1,

TCCCTGTATGTGCTCCAT (SEQ ID NO:3), directed against positions +1 to +18 relative to the start codon for the gene for rat C-raf-1,

AGGGGGGCCCCGTGGTGGCTCCAT (SEQ ID NO:4), directed against positions +1 to +24 relative to the start codon for the gene for A-raf-1,

GGGGCCCCGTGGTGGCTCCATGGA (SEQ ID NO:5), directed against positions -3 to +21 relative to the start codon for the gene for A-raf-1,

AGGGGGGCCCCGTGGTGGCTC (SEQ ID NO:6), directed against positions +4 to +24 relative to the start codon for the gene for A-raf-1,

GGGGCCCCGTGGTGGCTCCAT (SEQ ID NO:7), directed against positions +1 to +21 relative to the start codon for the gene for A-raf-1,

GCCCCGTGGTGGCTCCATGGA (SEQ ID NO:8), directed against positions -3 to +18 relative to the start codon for the gene for A-raf-1,

GCCCCGTGGTGGCTCCATGGAGCC (SEQ ID NO:9), directed against positions -6 to +18 relative to the start codon for the gene for A-raf-1,

GCACATGGGGACCCCCTC (SEQ ID NO:10), directed to an internal sequence of the gene for A-raf-1,

GCCCTCACACCACTGGGT (SEQ ID NO:11), directed to a consensus sequence of the raf gene family,

GCCCTCGCACCACTGGGT (SEQ ID NO:12), directed to a consensus sequence of the raf gene family,

CCGGCGCCGGCCTCGGGC (SEQ ID NO:13), directed to an internal sequence of the gene for B-raf-1, and

GCATTCTGCCCCCAAGGA (SEQ ID NO:14), directed to an internal sequence of the gene for C-raf-1.

In a preferred embodiment, the bases of the polynucleotide, e.g., SEQ ID NO:1, are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes. Exonuclease enzymes hydrolyze the terminal phosphodiester bond of a nucleic acid. Endonuclease enzymes hydrolyze internal phosphodiester bonds of a nucleic acid.

By replacing a phosphodiester bond with one that is resistant to the action of exonucleases or endonucleases, the stability of the nucleic acid in the presence of those exonucleases or endonucleases is increased. As used herein, psuedophosphate bonds include, but are not limited to, methylphosphonate, phosphomorpholidate, phosphorothioate, phosphorodithioate and phosphoroselenoate bonds. Additionally, exonuclease and/or endonuclease resistant polynucleotides can be obtained by blocking the 3′ and/or 5′ terminal nucleotides with substituent groups such as acridine.

Preferred psuedophosphate bonds are phosphorothioate bonds. The psuedophosphate bonds may comprise the bonds at the 3′ and or 5′ terminus, the bonds from about 1 to about 5 of the 3′ and/or 5′ terminus bases, or the bonds of the entire polynucleotide. A preferred polynucleotide with psuedophosphate bonds is one in which all of the bonds are comprised of psuedophosphate bonds.

DNA or RNA polynucleotides can be prepared using several different methods, as is well known in the

art. See, e.g., Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, New York (1990). The phosphoramidate synthesis method is described in Caruthers et al., Meth. Enzymol. 154:287 (1987); the phosphorothioate polynucleotide synthesis method is described in Iyer et al., J. Am. Chem. Soc. 112:1253 (1990).

The present invention is also directed to a pharmaceutical composition comprising a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf, dissolved or dispersed in a physiologically tolerable diluent. Preferably, the polynucleotide is from about 15 to about 25 nucleic acid bases in length, and more preferably about 20 nucleic acid bases in length.

The present invention includes one or more polynucleotides as described above formulated into compositions together with one or more non-toxic physiologically tolerable or acceptable diluents, carriers, adjuvants or vehicles that are collectively referred to herein as diluents, for parenteral injection, for oral administration in solid or liquid form, for rectal or topical administration, or the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenous, by intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray.

The compositions can also be delivered through a catheter for local delivery at the site of vascular damage, via an intracoronary stent (a tubular device composed of a fine wire mesh), or via a biodegradable polymer. The compositions may also be complexed to ligands, such as antibodies, for targeted delivery of

the compositions to the site of smooth muscle cell proliferation.

The compositions are preferably administered via parenteral delivery at the local site of smooth muscle cell proliferation. The parenteral delivery is preferably via catheter.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions can also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers or propellants as may be required. Ophthalmic formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

Actual dosage levels of active ingredients in the compositions of the present invention may be varied so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

The total daily dose of the compounds of this invention administered to a host in single or divided dose may be in amounts, for example, of from about 1 nanomol to about 5 micromols per kilogram of body weight. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of

administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

B. The Processes

The present invention is further directed to a process for inhibiting vascular smooth muscle cell proliferation that comprises contacting vascular smooth muscle cells whose growth is to be inhibited in an aqueous medium suitable for growth of those cells with an inhibition-effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf and maintaining said contact in said aqueous medium under biological culture conditions for a time period sufficient for the growth of the contacted cells to be inhibited.

As used herein, an "inhibition-effective amount" is that amount of a polynucleotide of the present invention which is sufficient for inhibiting the growth or killing a cell contacted with such a polypeptide. Means for determining an inhibition-effective amount in a particular subject will depend, as is well known in the art, on the nature of the polynucleotide used, the mass of the subject being imaged, whether killing or growth inhibition of the cells is desired, and the like.

Contact is achieved by admixing the composition with a preparation of vascular smooth muscle cells.

Biological culture conditions are those conditions necessary to maintain the growth and replication of the vascular smooth muscle cells in a normal, polynucleotide-free environment. These biological culture conditions, encompassing such factors

as temperature, humidity, atmosphere, pH and the like, must be suitable for the proliferation of vascular smooth muscle cells in the absence of polynucleotides so that the effects of such polynucleotides on relevant growth parameters can be measured.

A preferred polynucleotide useful in this process has the sequence shown in SEQ ID NO:1. A further preferred polynucleotide useful in this process links the bases of SEQ ID NO:1 by psuedophosphate bonds that are resistant to cleavage by exonuclease enzymes. Preferred psuedophosphate bonds are phosphorothioate bonds.

Further preferred polynucleotides have the sequences shown in SEQ ID NO:2 through 14.

The present invention is still further directed to a process for treating vascular smooth muscle cell proliferation that comprises administering to a host mammal in need of such treatment an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

A host mammal in need of the treatment of a process for the inhibition of vascular smooth muscle cell proliferation suffers from a disease state in which such proliferation is implicated. Such disease states include, but are not limited to, vascular stenosis, post-angioplasty restenosis (including coronary, carotid and peripheral stenosis), other non-angioplasty reopening procedures such as atherectomy and laser procedures, atherosclerosis, atrial-venous shunt failure, cardiac hypertrophy, vascular surgery, and organ transplant.

In a preferred embodiment, the polynucleotide as described above is dissolved or dispersed in a physiologically tolerable diluent.

The present invention is yet further directed to a process for treating a disease state involving the proliferation of vascular smooth muscle cells that comprises administering to a host mammal in need of such treatment an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

A disease state involving the proliferation of vascular smooth muscle cells include, but not limited to, vascular stenosis, post-angioplasty restenosis (including coronary, carotid and peripheral stenosis), other non-angioplasty reopening procedures such as atherectomy and laser procedures, atherosclerosis, atrial-venous shunt failure, cardiac hypertrophy, vascular surgery, and organ transplant.

The following examples further illustrate the invention and are not to be construed as limiting of the specification and claims in any way.

Examples

Materials and Methods

Antisense Polynucleotide Preparation

All reagents were from Eppendorf or Glen Research.

Antisense polydeoxynucleotide solid phase syntheses were performed on Millipore CPG columns using cyanoethyl phosphoramidite chemistry on an Eppendorf Synostat D300 DNA synthesizer replacing iodine by 3H-1,2-benzodithiol-3-one 1,1-dioxide (BDTD Beaucage reagent). The polynucleotide was cleaved from the solid support by incubation with 3 ml of fresh, concentrated (30%) ammonium hydroxide for 90 minutes. Cleavage was facilitated by mixing of the solution every 30 minutes with the help of two 5 ml slip-tip syringes.

The solution was collected in a screw-capped glass vial and deprotection was accomplished either at room temperature for 24 hours or at 55 °C for 5 hours. The contents were transferred to a 13x100 mm glass tube, chilled on ice and evaporated to dryness using a Savant Speed-Vac.

The polynucleotide was then dissolved in 1 ml of 0.1M triethylammonium acetate (TEAA), pH 7.0. The polynucleotide was detritylated and purified on a Rainin Dynamax C8 semipreparative column (10mm x 25cm, 5μm, 300 A). The mobile phases were (A): 0.1M TEAA, pH7.0, 5% acetonitrile; (B): 95% acetonitrile, 5% water; (C): 0.5% TFA in water. The column was developed at 2ml/min with the following gradient: 10% B in A, 10 min; 100% A, 4 min; 100% C, 8 min; 100% A, 8 min; 100% A to 45% B in 24 min. This procedure first separates the trityl-on full length polynucleotide from its failure sequences containing free hydroxyl groups and synthesis reagents. This is followed by the removal of 5'-DMT by 0.5% TFA. Finally the gradient resolved the desired detritylated sequence from other contaminants.

Absorbance was monitored at 260mm to identify factions containing the polynucleotide which was then evaporated. The polynucleotide was dissolved in 1 ml water, evaporated to remove volatile salts, and finally dissolved in 0.5 ml sterile, low TE (10mM Tris, 1mM EDTA, pH 7.5).

The polynucleotide concentration was determined by measuring the absorbance at 260 nm. Typical yields were 30-40%. The integrity of the polynucleotide was determined by polyacrylamide gel electrophoresis (PAGE; 20% polyacrylamide, 7M urea) and staining with 0.2% methylene blue.

Smooth Muscle Cell Isolation and Culture

Male Sprague-Dawley rats weighing 350-450 g were euthanized with carbon dioxide. The carotid arteries were removed and trimmed free of adventitia, nerve, and fat under a dissecting microscope. Arteries were cut into approximately $1mm^3$ pieces and placed in a 125 ml Erlenmeyer flask containing 0.67 ml/carotid artery of the following enzyme cocktail:

79.2 ml Hanks Balanced Salt Solution (Gibco; HBSS), 0.8 ml 0.2M $CaCl_2$ (Fisher), 0.286 g HEPES free acid (Calbiochem), 0.03 g trypsin inhibitor (Sigma; Type I-S; 10,000 units/mg), 0.16 g bovine serum albumin (Sigma; Fraction V), 600 units elastase (Sigma; Type II-A; 28 units/mg), 16,000 units collagenase (Worthington; CLS II; 353 units/mg) adjusted to pH 7.4, and 0.2μm filtered.

The flask was placed on an orbital shaker at 150 rpm at 37°C for 2-2.5 hr. The suspension was triturated vigorously and filtered through a 70 μm nylon cell strainer. The filtrate was then centrifuged at 400 x g for 10 min. The pellet was resuspended in 4 ml/carotid of the following media: 20% fetal bovine serum albumin (Hyclone; FBS); 2mM glutamine (Gibco); 100 units/ml penicillin G sodium (Gibco); 100 μg/ml streptomycin sulfate (Gibco); DMEM (Gibco).

The cell suspension from one carotid was then seeded into one T25 flask (Falcon) and maintained at 37°C in 5% CO_2.

Proliferation Assay

After 6-7 days, cells were rinsed twice with PBS (phosphate buffered saline) and harvested by the addition of 4 ml of 0.05% trypsin-EDTA (Gibco; 0.25% trypsin-EDTA) followed by incubation at 37°C for 3-5 min. The flask was rinsed with an additional 4 ml media

(DMEM, 20% PBS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin). The trypsinized cells and the rinse were combined and centrifuged at 400 x g for 10 min.

The supernatant was removed and 5 mls of fresh media was added to the pellet. The pellet was resuspended by vigorous trituration, and the number of cells was determined using a Coulter counter.

The cells were diluted to 3,500 cells/100 μl and, using a 12 channel digital micropipette, seed 100 μl/well of the cells were seeded in a 96 well (Falcon) flat-bottom, microtiter cell culture plate. The culture plate was then incubated at 37°C in 5% CO_2.

The following day, each well was rinsed twice with 100 μl PBS, and overlaid with 100 μl/well growth arrest media: 0.1% FBS (heat inactivated at 65°C for 45 min.); 2mM glutamine; 50 units/ml penicillin; 50 μg/ml streptomycin.

Four days later, the growth arrest media was removed. The cell number was determined (treatment day counts) using a Coulter counter by averaging the cell number from three wells. To the remaining wells was added 100 μl complete media (DMEM, 10% FBS/65°C inactivated, glutamine, pen/strep) without or with antisense polynucleotides and the plates were placed in an incubator at 37°C in 5% CO_2.

Three days later, the wells were rinsed twice with 100 μl each PBS. The cell number from 3 wells was again determined (assay day counts) using a Coulter counter. To the remaining wells was added 100 μl of 45 μg/ml calcein A-M (Molecular Probes) in PBS. The plates were incubated for 1 hr. at 37°C.

After incubation, fluorescence was determined using a Cytofluor 2350 (Millipore) microtiter plate reader with excitation at 485 nm and emission at 530 nm.

Growth in cell number was calculated by subtracting treatment day cell counts from assay day cell counts. Based on the established linear relationship between fluorescence and cell number, the percent growth inhibition by the antisense polynucleotides was determined.

1. SMC Proliferation Assay

Carotid arteries were dissected from male Sprague-Dawley rats weighing 200 to 300 grams. Approximately 1 mm^3 minces were incubated in Dulbecco's minimal essential medium (DME) supplemented with 20 percent fetal bovine serum. The medium was changed every three to four days.

After 2 to 2.5 weeks of growth, trypsin was added to the growth culture to isolate cells which had grown out of the arterial explant. These isolated cells were plated in 96 well trays at a concentration of 2,500 cells per well. After one day of growth under the conditions described above, the cells were washed twice with 100 μl of phosphate buffered saline and placed in growth arrest medium consisting of DME supplemented with 0.5% heat-inactivated fetal bovine serum.

After four days in growth arrest medium, cell number was determined by a fluorescence-based cell proliferation assay using calcein-AM (Molecular Probes; Eugene, OR). The medium was removed and triplicate wells were incubated with 1 mM calcein-AM, dissolved in phosphate buffered saline, for 1 hour at 37°C. Fluorescence was determined using a Cytofluor plate reader (Millipore; Boston, MA), at 580 nm following excitement at 450 nm. Under the cell culture conditions used, there was a linear relationship between cell number (determined by Coulter counting) and fluorescence. Additional sets of triplicate wells were

either untreated (controls) or treated with the indicated concentrations of polynucleotides dissolved in DME with 10% serum heat-inactivated at 65°C. After an additional three days, fluorescence was again determined using calcein-AM, as described above.

2. Polynucleotide Effects on SMC Proliferation

The proliferation of smooth muscle cells according to the assay described in Example 1 was determined in the presence of antisense polynucleotides which hybridized to a portion of human A-raf-1 as well as an antisense polynucleotide directed against plasminogen activator inhibitor-1 (PAI-1), as a negative control.

The results of these assays are shown in Figure 1. The data in the bar graph is depicted to indicate that 100% inhibition reflects the absence of SMC proliferation during the assay. Values of less than 0% indicate that the treated cells proliferated to a greater extent than did untreated cells. Values of greater than 100% means that there were fewer cells at the end of the assay than at the beginning.

The data show that 50 μM of the antisense polynucleotide directed against A-raf-1 showed approximately 100 percent inhibition of SMC proliferation, compared to about 10% inhibition by the control polynucleotide.

3. Polynucleotide Effects on Human SMC Proliferation

In order to evaluate growth regulated expression of raf mRNAs in humans, SMCs were isolated from normal human aorta or diseased human carotid artery endarterectomy specimens by enzymatic dissociation with collagenase and elastase followed by culture in 10%

fetal bovine serum in DMEM. After 5-7 days, cells were plated at 40,000 cells/100mm^2 dish. The following day cells were growth stimulated in 10% FBS in DMEM or growth arrested in 0.1% FBS in DMEM. Three days later, RNA was isolated and mRNA levels determined by using the reverse transcriptase polymerase chain reaction (RT-PCR).

As shown in Table 1 A raf mRNA levels in both cell types were elevated in arrested cells and not further induced by serum treatment. mRNA for B raf and C raf were low or undetectable in arrested cells and induced by serum treatment. Thus SMCs derived from the vessel wall express all three members of the raf gene family under growth conditions similar to those after angioplasty.

Table 1

	Normal Aorta		Carotid Endarterectomy*	
mRNA	Log	Arrest	Log	Arrest
GAPDH	+++++	+++++	+++++	+++++
A raf	++++	++++	++++	++++
B raf	++	-	+	-
C raf	++	-	+++	+
PCNA	++	-	+	-

*The pluses represent relative band intensities of the PCR amplified products as seen on agarose gels. The minuses indicate the absence of a detectable band.

Since abnormal SMC growth stimulation is a serious consequence of angioplasty that often leads to restenosis in humans, mRNAS for raf gene family members were tested for overexpression in response to the growth stimulatory effects of angioplasty. For these experiments, the rat carotid artery balloon angioplasty model of restenosis. At various times after angioplasty, arteries were removed, trimmed of

adventitia and nerve tissue, and mRNA analyzed by RT-PCR.

Table 2 shows that A raf was expressed in the normal quiescent state and further induced in response to angioplasty. This is analogous to regulation of human cells in culture described above. Expression of B raf and C raf was elevated within 6 hr post-angioplasty. As indicated by PCNA expression, this is the time when medial SMCs are proliferating.

Table 2

Time Course of mRNA Expression in Response to Balloon Angioplasty of Rat Carotid Artery

		Time Post-Angioplasty											
mRNA	control	2hr	4hr	6hr	1d	2d	4d	7d		10d		14d	
								int	med	int	med	int	med
GAPDH	++	++	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
A raf	++	++	++	++	+++	+++	+++	+++	++	+++	++	+++	++
B raf	-	-	-	+	++	++	+	+	++	+	++	+	+
C raf	+	-	+	++	++	++	++	++	+	+++	++	+++	++
PCNA	-	-	-	+	+++	++++							++

Values represent relative band intensities of PCR amplified products on gels.

When SMCs were isolated from ballooned arteries and analyzed for mRNA expression as shown in Table 3, this alteration of phenotype of overexpression of raf mRNAs was found to be maintained in cell culture and was higher in SMCs from the neointima than from the underlying media. As evidenced by higher PCNA expression, neointimal cells were also more actively proliferating than medial cells. Thus, angioplasty of the rat carotid artery increased expression for the A, B, and C raf mRNAs. This induction was not only maintained in culture but enriched in the population of abnormally proliferating SMCs that constitute the neointima.

Table 3

mRNA	Neointima	Media
GAPDH	+++++	+++++
A raf	++	+
B raf	+++	+
C raf	+++	+
PCNA	++++	+++

Table 4 shows that antisense to the human A raf, B raf and rat C raf (identical to the human gene) mRNAs exhibit significant growth inhibitory properties. Sequence optimization studies were then performed to identify the most important region for antisense targeting of the A raf mRNA in SMCs from the rat carotid artery. Table 4 illustrates the positions and sequences of the antisense oligos tested in addition to their efficacy of growth inhibition. SEQ ID NO:4, which stretches from +1 to +24 relative to the start site, was the most potent. Antisense to some internal sequences such as SEQ ID NO:10 were also very effective at inhibiting growth.

Table 4

Position	SEQ ID NO	% Growth Inhibition
	Human A-raf-1 Sequences	
+1/+24	4	77
+1/+18	1	71
-3/+21	5	62
+4/+24	6	59
+1/+21	7	58
-3/+18	8	56
-6/+18	9	44
	Internal Human A-raf-1 Sequences	
	10	72
	11	55
	12	49
	Human B-raf-1 Sequences	
+1/+18	2	73
Internal	13	45
	Human C-raf-1 Sequences	
+1/+18	3	65
Internal	14	52

In order to determine if a relationship existed between SMC growth and raf mRNA expression, we tested the effects of antisense oligonucleotide to these genes on the growth of SMC isolated from normal human aorta. As shown in Table 5, antisense to A raf (SEQ ID NO:10) inhibited SMC growth and down regulated A raf mRNA while not affecting expression of the genes for GAPDH, B raf or C raf. Antisense to the gene for B raf inhibited growth, down regulated the target mRNA, and did not affect expression of GAPDH. Antisense to C raf inhibited growth and target mRNA induction but not A raf or GAPDH expression.

Table 5

Antisense Treatment	% Growth Inhibition	mRNA GAPDH	A raf	B raf	C raf
None	0	+++++	+++	++	+++
A raf	68	+++++	+	++	+++
B raf	62	+++++	n.d.*	+	n.d.*
C raf	71	+++++	+++	n.d.*	+

*n.d.=Not determined.

In summary, the data show that SMCs cultured from human carotid endarterectomy specimens and normal human aorta overexpressed raf gene family mRNAs in log-phase growth compared to arrested, quiescent cells. The data also show that raf mRNA expression was elevated in response to angioplasty of the rat carotid artery. This elevated expression was maintained in SMC cultured from the neointima and media that formed two weeks after angioplasty. Since angioplasty induced raf mRNAs and SMC growth, and since growth-stimulated SMCs from diseased patients express elevated raf mRNAs, raf levels are crucial to the abnormal proliferative response that leads to intimal thickening and restenosis. In order to test this hypothesis, an antisense strategy to inhibit expression was developed. In cell culture, raf antisense specifically inhibited proliferation of SMCs from normal rat carotid artery. Sequences around the translation initiation site that were found to be optimal for this inhibition resulted in down regulation of the cognate mRNA. These antisense polynucloetides also inhibited proliferation and down regulated the mRNA of cultured SMCs from normal human aorta specimens.

The foregoing specification, including the specific embodiments and examples is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Denner, Larry A
Rege, Ajay A
Dixon, Richard AF

(ii) TITLE OF INVENTION: ANTISENSE MOLECULES DIRECTED AGAINST GENES OF THE RAF ONCOGENE FAMILY

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Katz, Martin L.
(B) REGISTRATION NUMBER: 25,011

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (312)616-5400
(B) TELEFAX: (312)616-5460

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCCCCGTGGT GGCTCCAT 18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACCGCTCAGC GCCGCCAT 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCCTGTATG TGCTCCAT 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGGGGGGCCC CGTGGTGGCT CCAT 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGCCCCGT GGTGGCTCCA TGGA 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGGGGGGCCC CGTGGTGGCT C 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGGCCCCGT GGTGGCTCCA G 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCCCGTGGT GGCTCCATGG A 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCCCGTGGT GGCTCCATGG AGCC 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCACATGGGG ACCCCCTC 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCCTCACAC CACTGGGT 18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCCTCGCAC CACTGGGT 18

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGGCGCCGG CCTCGGGC 18

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCATTCTGCC CCCAAGGA 18

CLAIMS

1. A polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

2. The polynucleotide of claim 1 wherein said gene for raf selected from the group consisting of A-raf-1, B-raf-1 and C-raf-1.

3. The polynucleotide of claim 1 wherein said gene for raf is human raf.

4. The polynucleotide of claim 1 that is an antisense molecule having the sequence shown in SEQ ID NO:1 through 14.

5. The polynucleotide of claim 1 wherein the bases of said polynucleotide are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

6. The polynucleotide of claim 5 wherein said bonds are phosphorothioate bonds.

7. A pharmaceutical composition comprising a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf, dissolved or dispersed in a physiologically tolerable diluent.

8. The pharmaceutical composition of claim 7 wherein the polynucleotide that is an antisense molecule having the sequence shown in SEQ ID NO:1 through 14.

9. The pharmaceutical composition of claim 7 wherein the bases of said polynucleotide are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

10. The pharmaceutical composition of claim 9 wherein said bonds are phosphorothioate bonds.

11. A process for inhibiting vascular smooth muscle cell proliferation that comprises contacting vascular smooth muscle cells whose growth is to be inhibited in an aqueous medium suitable for growth of those cells with an inhibition-effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf and maintaining said contact in said aqueous medium under biological culture conditions for a time period sufficient for the growth of the contacted cells to be inhibited.

12. The process of claim 11 wherein said polynucleotide has the sequence shown in SEQ ID NO:1 through 14.

13. The process of claim 11 wherein the bases of said polynucleotide are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

14. The polynucleotide of claim 13 wherein said bonds are phosphorothioate bonds.

15. A process for treating vascular smooth muscle cell proliferation that comprises administering to a host mammal in need of such treatment an effective

amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

16. The process of claim 15 wherein said polynucleotide is dissolved or dispersed in a physiologically tolerable diluent.

17. The process of claim 15 wherein said polynucleotide has the sequence shown in SEQ ID NO:1 through 14.

18. The process of claim 15 wherein the bases of said polynucleotide are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

19. The process of claim 18 wherein said bonds are phosphorothioate bonds.

20. The process of claim 15 wherein said administering is to the local site of said proliferation.

21. A process for treating a disease state involving the proliferation of vascular smooth muscle cells that comprises administering to a host mammal in need of such treatment an effective amount of a polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the gene for raf.

22. The process of claim 21 wherein said disease state is selected from the group consisting of vascular stenosis, post-angioplasty restenosis

(including coronary, carotid and peripheral stenosis), other non-angioplasty reopening procedures such as atherectomy and laser procedures, atherosclerosis, atrial-venous shunt failure, cardiac hypertrophy, vascular surgery, and organ transplant.

23. The process of claim 21 wherein said polynucleotide is dissolved or dispersed in a physiologically tolerable diluent.

24. The process of claim 21 wherein said polynucleotide has the sequence shown in SEQ ID NO:1 through 14.

25. The process of claim 21 wherein the bases of said polynucleotide are linked by psuedophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

26. The process of claim 25 wherein said bonds are phosphorothioate bonds.

27. The process of claim 21 wherein said administering is to the local site of said proliferation.

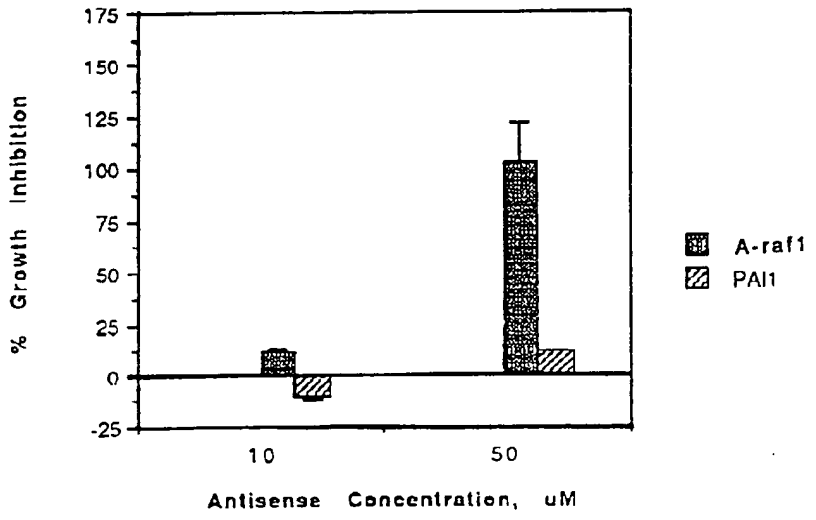
28. A polynucleotide of about 10 to about 50 nucleic acid bases in length, said polynucleotide hybridizing to the about 5 to about 25 nucleic acid bases flanking the start codon of the mRNA for raf.

29. The polynucleotide of claim 28 wherein said mRNA for raf is human raf.

30. The polynucleotide of claim 28 that is an antisense molecule having the sequence shown in SEQ ID NO:1 through 9.

31. The polynucleotide of claim 28 wherein the bases of said polynucleotide are linked by pseudophosphate bonds that are resistant to cleavage by exonuclease or endonuclease enzymes.

FIGURE 1


175
150
125
100
75
50
25
0
-25
% Growth Inhibition
10
50
Antisense Concentration, uM
A-raf1
PAI1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12603

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 48/00; C07H 21/04
US CL :514/44; 536/24.5; 935/33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 536/24.5; 935/33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DNA Databases: GenBank and EMBL, CA, MEDLINE, EMBASE, LIFESCI BIOSIS DERWENT, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 266, No. 27, issued 25 September 1991, Chiang et al., "Antisense Oligonucleotides Inhibit Intercellular Adhesion Molecule 1 Expression by Two Distinct Mechanisms," pages 18162-12171, see entire document.	1-3, 5, 6 --------- 4, 7-31
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 188, No. 1, issued 15 October 1992, Duff et al., "Angiotensin II Stimulates the pp44 and pp42 Mitogen-activated Protein Kinases in Cultured Rat Aortic Smooth Muscle Cells," pages 257-264, see entire document.	4, 7-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 19 March 1994	Date of mailing of the international search report 11 APR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SCOTT HOUTTEMAN Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12603

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Vol. 88, issued September 1991, Agrawal et al., "Pharmacokinetics, Biodistribution, and Stability of Oligodeoxynucleotide Phosphorothioates in Mice," pages 7595-7599, see entire document.	4, 7-31
Y	NUCLEIC ACIDS RESEARCH, Vol. 14, No. 2, issued 1986, Bonner et al., "The Complete Coding Sequence of the Human *raf* Oncogene and the Corresponding Structure of the *c-raf*-1 Gene," pages 1009-1015, see entire document.	4, 7-31